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Adventitious shoots induction and plant regeneration from cotyledons of watermelon (*Citrullus lanatus* L.)

Xiaoqiang Zhao, Xiaowei Niu and Min Fan*

Institute of vegetable, Zhejiang Academy of Agricultural Science, Hangzhou 310021, China.

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A highly efficient regeneration system is a prerequisite step for successful genetic transformation of watermelon cultivars (*Citrullus lanatus* L.). The objective of this study was to establish efficient *in vitro* plant regeneration for three watermelon cultivars. To achieve optimal conditions for adventitious shoot induction, the 5-day-old explants (cotyledon base portion, apical portion and hypocotyl) of three cultivars were placed on MB_5 media supplemented with different concentrations and combinations of growth regulators (1.0 to 10.0 mg L^{-1} 6-benzyladenine (BA) and 0 to 1.0 mg L^{-1} indole acetic acid (IAA)); the explants from seedling of different development stages (0 to 10 d) were cultured on MB_5 medium containing 2.0 mg L^{-1} BA and 0.2 mg L^{-1} IAA for investigating the effect of age on adventitious shoots initiation; besides, 5-day-old seedlings were grown on optimal regeneration medium supplemented with different concentrations of kanamycin for screening the lowest lethal concentration for adventitious shoots. The results show that the basal region of cotyledon showed higher frequency of shoot formation (79.17-83.33%) than the apical region (5.23-8.25%); high percentage of shoots regeneration was induced from 5-day-old cotyledons base portion cultured on MB_5 containing 1 or 2 mg L^{-1} BA; the 100 mg L^{-1} kanamycin proved to be the optimal concentration for screening the transformants. Our results provide an efficient stable regeneration system for genetic transformation of watermelon.

Key words: Watermelon (*Citrullus lanatus*), cotyledon, growth regulator, kanamycin, regeneration.

INTRODUCTION

Watermelon (*Citrullus lanatus* L.), one of the most important vegetable crops, is eaten chiefly as a fresh fruit, because of its rich carbohydrates, vitamins and minerals (Cho et al., 2008; Huang et al., 2011; Yu et al., 2011; Guo et al., 2013). It originated from tropical and subtropical Africa (Cho et al., 2008). However, it is susceptible to a number of diseases (Kim et al., 1998; Compton and Gray, 1999), insect pest (Coffey et al., 2015) and other environmental factors, which lead to reductions in crop yield and quality. To decrease the

*Corresponding author. E-mail: watermelonresearch@163.com, fanminfm@sina.com. Tel: +86-571-86416057.

#Xiaoqiang Zhao and Xiaowei Niu contributed equally to this article.

Abbreviations: MS, Murashige and Skoog; **MB_5,** MS salts, B5 vitamins; **BA,** 6-benzyladenine; **IAA,** indole acetic acid.

effects of these disadvantage factors, annual field rotations, frequent chemical sprays, and new elite cultivars (Compton and Gray, 1999) are required. The annual field rotation needs lots of land, and the chemical sprays can cause environmental pollution. Thus, breeding elite cultivars of watermelon is the most effective way to improve crop yield and quality.

Although removing unfavorable traits and finding material sources through traditional breeding methods are genetically agreeable (Compton and Gray, 1993a), introducing resistant genes into commercial cultivars by traditional breeding mechanisms is not very efficient. Therefore, introduction of foreign genes has a potential for the improvement of watermelon. Recently, success of transformation has been reported in watermelon using transgenic technology (Choi et al., 1994; Chen et al., 1998; Ellul et al., 2003; Akashi et al., 2005; Park et al., 2005; Cho et al., 2008; Huang et al., 2011; Yu et al., 2011; Lin et al., 2012). Furthermore, the success of genetic manipulation using transgenic technology mainly depends on an efficient regeneration system.

Plant regeneration is a prerequisite step for genetic transformation and is usually influenced by biotic factors such as genotype and explant type, and abiotic factors such as culture media and environmental conditions (Liu et al., 2010). Most of the regeneration occurs via organogenesis, and successful plant regeneration from watermelon mature cotyledons (Srivastava et al., 1989; Dong and Jia, 1991; Compton and Gray, 1993b; Tabei et al., 1993; Chaturvedi and Bhatnagar, 2001; Pirinç et al., 2003; Li et al., 2011; Zhang et al., 2014), immature cotyledons (Compton and Gray, 1993a), petiole (Jeyakumar et al., 2014) and hypocotyl (Srivastava et al., 1989) have been reported. However, these reports of shoot regeneration from watermelon cotyledons are contradictory with regards to genotype, age of explant, optimum growth regulator combinations and concentration, conditions of rooting and acclimatization, and may not be suitable for many commercial cultivars. Therefore, it is necessary to establish an efficient protocol for regeneration for a wide range of commercial cultivars.

The objectives of this study were to establish efficient plant regeneration system by testing the effects of the type and age of the explants and growth regulator concentrations on plant regeneration in three commercial cultivars. This tissue culture system would be used for the transformation of watermelon by *Agrobacterium tumefaciens*.

MATERIALS AND METHODS

Plant materials and adventitious shoots introduction

Three watermelon (*Citrullus vulgaris* L.) cultivars, 'Heihaier', 'Tiancheng' and 'Jinmanduo', were used throughout this study. After the mature seeds coats were removed by a forceps, the seeds were surface-sterilized in 70% (v/v) ethanol (Shenggong China) for 1 min, in 0.1% (m/v) $HgCl_2$ (Shenggong China) for 5 min, and then washed 3-5 times in sterilized distilled water. The sterilized decoated seeds were placed on ½ strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) under dark conditions at 25 ± 1°C for germination. The hypocotyls and cotyledons were removed by a scalpel from 5-day-old seedlings and then the cotyledons were cut crosswise and lengthwise into cotyledon base portion and apical portion. The different parts of cotyledons and the hypocotyls segments were set as explants. Then, explants were placed on MB_5 media [MS salts, B5 vitamins (Gamborg et al., 1968), 30 g L^{-1} sucrose (Shenggong China) and 7 g L^{-1} agar (Shenggong China)] supplemented with 1, 2, 3, 4, 5, 6, 7, and 10 mg L^{-1} N6-benzyladenine (BA, Invitrogen USA) in combination with 0, 0.2, 0.5, and 1 mg L^{-1} indole-3-acetic (IAA, Invitrogen USA) for shoot induction.

Optimal seedling age for adventitious shoots regeneration

To investigate the effect of age of the seedlings on adventitious shoots initiation, the basal region of cotyledon of three cultivars from seedling of different ages (0, 3, 5, 7 and 10 days) were cultured on MB_5 medium containing 2 mg L^{-1} BA and 0.2 mg L^{-1} IAA.

Rooting and plant acclimatization

After four weeks in shoots regeneration media, regenerated multiple shoot buds from cotyledon base portion were sub cultured onto MB_5 medium supplemented with 2.0 mg L^{-1} BA, 1.0 mg L^{-1} IAA and 2.0 mg L^{-1} Gibberellic acid (GA_3, Invitrogen USA) for shoot elongation. The resulting elongated shoots (about 2-2.5 cm) were separated into signal one and transferred to rooting medium, which consisted of ½ strength MS medium supplemented with 0.2 mg L^{-1} a-naphthalene acetic acid (NAA, Invitrogen USA). Regenerated plantlets with well-developed roots were gently washed with tap water to remove the attached medium from their roots and then transplanted to flowerpots (14 × 16.5 cm, Shenggong China) in greenhouse, one plant per pot. All pots contained equal quantities of the nutrient soil (1 vermiculite: 1 perlite: 2 garden nutrient soils, by volume).

Determination of optimal kanamycin concentrations for shoot bud induction

To determine the lowest lethal concentration of kanamycin (Invitrogen USA) for adventitious shoot induction, the cotyledon base portion of 5-day-old seedlings was used as explants. The explants of three cultivars were grown on optimal regeneration medium supplemented with different concentrations of kanamycin (0, 50, 75, 100 and 125 mg L^{-1}). The explants were maintained for four weeks without subculture.

For all *in vitro*, the pH of the medium was adjusted to 5.85 before autoclaving; the medium was then autoclaved for 20 min at 1.1 kg cm^{-2} and 121°C. Cotyledon were cultured in petri plates (90×16 mm) containing 20-25 ml medium for shoot induction and adventitious shoots were cultured in culture bottle (90×60 mm) that contained 30 ml medium and five adventitious shoots for elongation and rooting. The cultures were maintained at 24 ± 1°C under a 16 h photoperiod of 40-50 µmol· m^{-2} s^{-1} irradiance provided by cool white fluorescent lamps. Cultures were observed weekly, and the adventitious bud primordia and shoots were counted at the same time. Each treatment was arranged in a completely randomized design (CRD) with 4 replicates and 8 explants per replicate. The number of shoot and roots initiated per explants were calculated after 1 month. The data were analyzed by analysis of variance (ANOVA) and Duncan's multiple range tests ($p< 0.05$) with the SPSS software (SPSS Inc, Chicago, IL, USA).

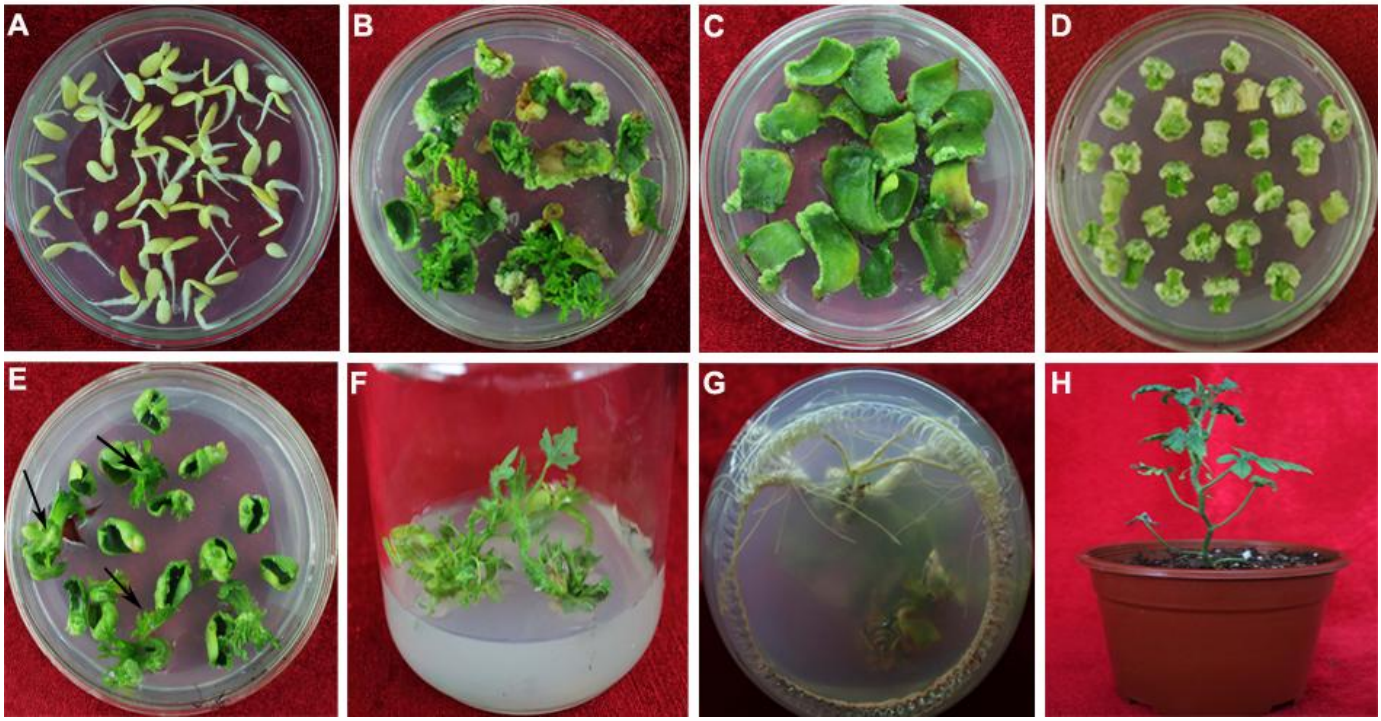

Figure 1. Plant regeneration from cotyledon explants of watermelon (*Citrullus lanatus* L.). **A.** Five-day-old germinated seeds under dark conditions; **B.** Multiple shoots regeneration from the wound base portion of cotyledon; **C.** Calli from wound apical portion of cotyledon; **D.** Calli from wound site of hypocotyl; **E.** Hyperhydric shoots formation from the wound base portion of cotyledon (Arrows indicate hyperhydric shoot); **F.** Multiple shoots elongated on the shoot elongation medium; **G.** Elongated shoots rooted on the rooting medium; **H.** Plantlet growing in greenhouse.

RESULTS

Shoot regeneration

The sterilized decoated seeds were cultured on ½ strength MS medium under dark conditions for 5 days and then the radicles (Figure 1A) were carefully removed. Cotyledons and hypocotyls on regeneration medium expanded in size during the first few days of culture. There were significant differences in shoot induction of different regions of cotyledons and hypocotyls. Green meristematic protrusions, which resembled young shoot apices, were observed at the enlarged cotyledon base portion. Then multiple shoot buds differentiated directly from per cotyledon base portion within 10-20 days (Figure 1B). Calli were present in all cotyledons apical portion of three cultivars in induction medium (Figure 1C), but only 5.23-8.25% shoot buds differentiated from callus. Analysis showed that the cotyledon apical portion gave the lower frequency of shoots induction for 'Heihaier', 'Tiancheng' and 'Jinmanduo' (5.25, 5.23 and 8.25%, respectively). However, white loose calli were formed from the hypocotyls but no shoot buds differentiated from callus (Figure 1D). At the same time, some regenerated shoot from cotyledon base portion appeared to have a hyperhydric phenotype (Figure 1E).

Effect of genotypes on shoot induction

The regenerated shoots were obtained from the three cultivars. The cotyledon base portions of the three watermelon genotypes gave shoot differentiation frequencies of 8.33-83.33% on MB_5 medium supplemented with BA in combination with IAA (Figure 2). The highest shoot induction frequencies were the same for 'Heihaier' (83.33%) and 'Tiancheng' (83.33%) but these were higher than 'Jinmanduo' (79.2%) and they were no significant differences in shoot induction. The genotypes also differed in the time required for the initiation of shoot buds and the number of shoots produced. The cotyledons from 'Heihaier' and 'Jinmanduo' showed faster morphological response than the cotyledons from 'Tiancheng'.

Effect of BA and IAA concentration on shoot induction

The cotyledon base portion of 'Heihaier' gave high shoot regeneration frequencies on MB_5 medium containing 2.0 mg L^{-1} BA or 2.0 mg L^{-1} BA and 0.2 mg L^{-1} IAA (83.33

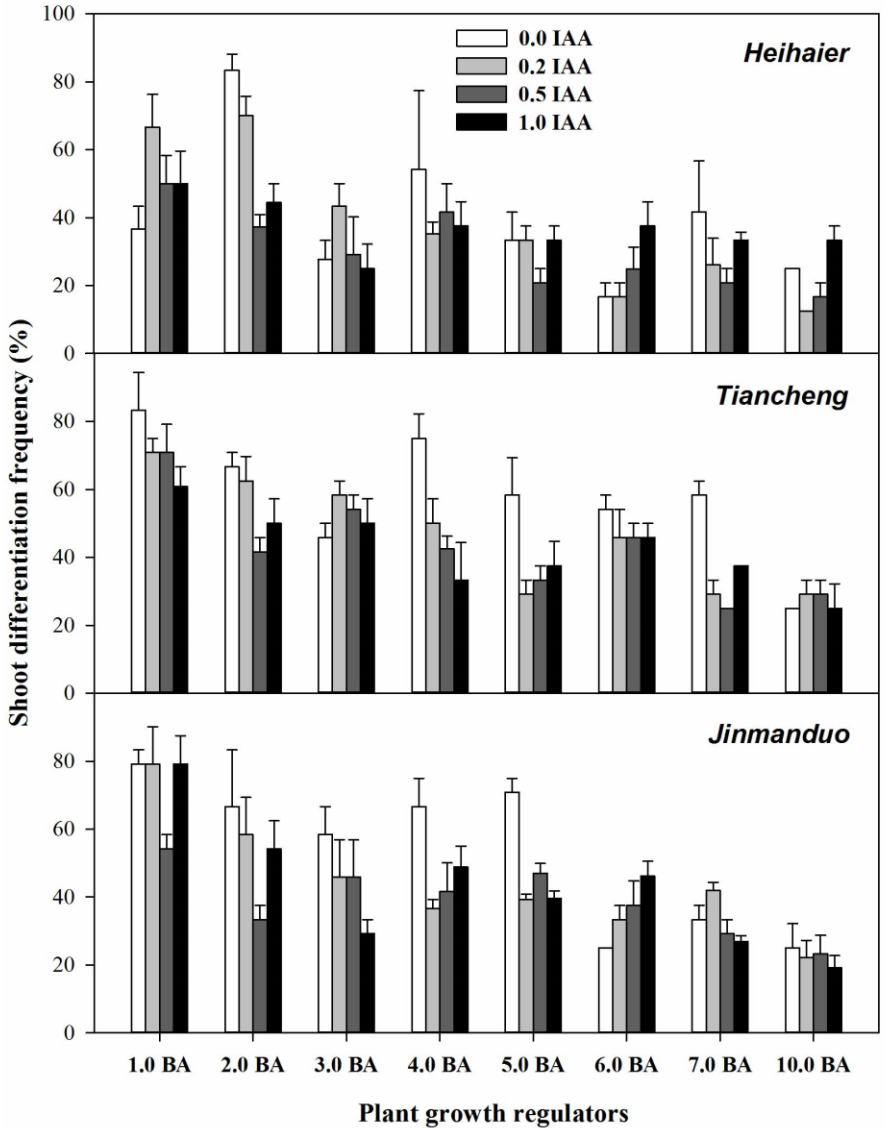

Figure 2. Effects of different concentrations of BA and IAA on adventitious shoots formation from cotyledon base portion explants of the three watermelon cultivars (*Citrullus lanatus* L.). Adventitious shoots of three watermelon cultivars. The shoot differentiation frequency is expressed as the percentage of the cotyledon base portion forming shoots in 5 weeks. Error bars with standard error.

and 70.0%, respectively). Low concentrations of BA (1.0-3.0 mg L^{-1}) in shoot induction medium were effective for shoot initiation and small multiple buds were usually formed on medium which contained low concentrations of IAA (0.2 mg L^{-1}). However, shoot regeneration with high concentrations of BA (4.0-10.0 mg L^{-1}) showed lower frequencies (16.67-54.17%) than for low concentrations of BA, whether we added the IAA or not, and the number of shoot buds per explant also decreased. Furthermore, these shoots were difficult to elongate. 'Tiancheng' and 'Jinmanduo' showed similar responses to the BA in combination with IAA, and the optimal shoot regeneration medium are MB_5 medium supplied with 1 mg L^{-1} BA (Figure 2).

Effect of age of donor seedlings on shoot induction

The shoot differentiation frequencies of 'Heihaier', 'Tiancheng' and 'Jinmanduo' varied remarkably with the different ages of seedlings at MB_5 medium containing 2 mg L^{-1} BA and 0.2 mg L^{-1} IAA. The shoot differentiation frequencies of three varieties ('Heihaier', 'Tiancheng' and 'Jinmanduo') increased with extension of culture time (0, 3 and 5 days); 5-day-old seedlings had the highest frequencies, which were 1.28-, 2.55- and 1.25-folds of 3-day-old seedlings, respectively.

However, shoot regeneration ability of the cotyledonary explants decreased dramatically when the age of donor seedlings exceeded seven days (Table 1). At the same

Table 1. Effect of age of cotyledon base portion explants on shoot induction of three watermelon cultivars (Citrullus lanatus L.).

Cultivar	Age of explants (d)	No. of explants cultured	No. of explants forming adventitious buds	Induction rate (%)
	0	32	10	31.25 ± 3.61 c
	3	32	18	56.25 ± 3.61 b
'Heihaier'	5	32	25	71.88 ± 3.13 a
	7	32	17	53.13 ± 5.98 b
	10	32	6	18.75 ± 3.61 d
	0	32	6	18.75 ± 3.61 c
	3	32	9	28.13 ± 3.13 c
'Tiancheng'	5	32	23	71.87 ± 3.13 a
	7	32	15	46.88 ± 3.13 b
	10	32	6	18.75 ± 3.61 c
	0	32	11	34.38 ± 3.13 c
	3	32	20	62.50 ± 5.10 b
'Jimanduo'	5	32	25	78.13 ± 3.13 a
	7	32	16	50.00 ± 5.10 b
	10	32	8	25.00 ± 5.10 c

Date represent mean ± standard error for four replicates (sixteen explants each). Values with the different lowercase letters within each variable have significant difference at $p < 0.05$ (Duncan's multiple range test).

time, with the increase of seedling age (more than seven days), the number of adventitious buds derived from every explant also decreased gradually (less than 3). These results indicate that the selection of younger aseptic seedling was one of the keys to increasing the budding rate. Thus, 5-day-old seedlings proved to be the optimal source for culture.

Shoot elongation

It was important that the shoot buds were transferred to elongation medium in time. Shoot buds could elongate normally when they were subcultured on MB_5 medium supplied with 2 mg L^{-1} BA, 1 mg L^{-1} IAA and 2 mg L^{-1} GA_3 after four weeks on regeneration medium (Figure 1F). If the cotyledons with multiple shoot buds were maintained on regeneration medium for longer than four weeks, only a few shoot buds could elongate and the rest of shoot buds showed abnormal morphology, such as yellow color, swollen and brittle buds, and some callus formed on the wound. At the same time, about 3-6 shoots can be obtained from each explants of the cultivars used in this study.

Rooting of shoot

For the induction of root, elongated shoots from the three cultivars were excised and cultured on ½ strength MS medium supplemented with 0.2 mg L^{-1} NAA. The roots were observed after about two weeks (Figure 1G). In rooting medium, 94.8 (55/58), 95 (57/60) and 94.3% (50/53) of green shoots from 'Heihaier', 'Tiancheng' and 'Jinmanduo' rooted. After three weeks, rooted plantlets were transferred to greenhouse, only 70% of the plantlets survived and their leaves turned dark green in one week (Figure 1H).

Effects of kanamycin on adventitious buds induction

Kanamycin had a great influence on organ differentiation for watermelon cotyledon (Figure 3). Adventitious buds from three watermelon cotyledon base portion were insensitive to low concentrations (0-75 mg L^{-1}) of kanamycin, and the range of induction rate for 'Heihaier', 'Tiancheng' and 'Jinmanduo' were 15.63-71.88, 18.75-71.88 and 15.63-78.13%, respectively. However, the frequency of shoot regeneration decreased rapidly with increasing kanamycin concentration and the shoots were not formed from cotyledon base portion cultured at 100 mg L^{-1} kanamycin (Table 2). Undifferentiated cotyledon became yellow and necrosis after 1 month.

DISCUSSION

Three cultivars received positive results on MB_5 medium supplemented with low BA concentration alone (1.0 or

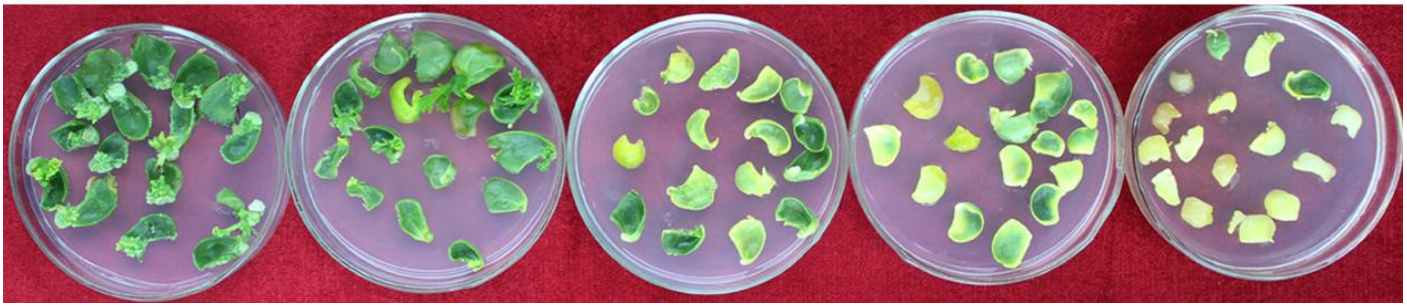

Figure 3. Effect of different concentrations of kanamycin on adventitious shoots formation from cotyledon base portion explants of the three watermelon cultivars (*Citrullus lanatus* L.). Adventitious shoot was determined after 4 weeks of cultivation on induction medium. The antibiotic concentrations were 0, 50, 75, 100 and 125 mg L^{-1} kanamycin from left to right.

Table 2. Effect of kanamycin concentrations on shoot regeneration from cotyledon base portion explants of three watermelon cultivars (*Citrullus lanatus* L.)

Cultivar	Kanamycin concentrations (mg L^{-1})	No. of explants cultured	No. of explant forming adventitious buds	Induction rate (%)
'Heihaier'	0	32	25	71.88 ± 3.13 a
	50	32	9	28.13 ± 3.13 b
	75	32	5	15.63 ± 3.13 c
	100	32	1	3.13 ± 3.13 d
	125	32	0	0 ± 0 d
'Tiancheng'	0	32	23	71.88 ± 3.13 a
	50	32	10	31.25 ± 3.61 b
	75	32	6	18.75 ± 3.61 c
	100	32	1	3.13 ± 3.13 d
	125	32	0	0 ± 0 d
'Jimanduo'	0	32	25	78.13 ± 3.13 a
	50	32	15	46.88 ± 5.98 b
	75	32	5	15.63 ± 3.13 c
	100	32	0	0 ± 0 d
	125	32	0	0 ± 0 d

Values with the different lowercase letters within each variable have significant difference at $p < 0.05$ (Duncan's multiple range test). Note: Date represent mean ± standard error for four replicates (eight explants each).

2.0 mg L^{-1}). This phenomenon suggests that low concentration of BA is necessary for shoot induction and differentiation from the cotyledon of watermelon. Srivastava et al. (1989) induced adventitious shoots from cotyledon in watermelon by low BA concentration (1.0 mg L^{-1}) and similar results were reported in many studies (Compton and Gray, 1993b; Choi et al., 1994; Pirinç et al., 2003; Huang et al., 2011; Yu et al., 2011; Choi et al., 2012). These reports showed that the high frequency of shoot regeneration in watermelon required a low concentration of BA (BA: 1.0-4.0 mg L^{-1}). However, Blackmom and Reynold (1982) induced adventitious shoots from cotyledon of watermelon by using the combination of 10 mg L^{-1} 2ip and 0.1 mg L^{-1} NAA, and similar results were reported (Dong and Jia, 1991; Tabei et al., 1991). They thought high concentration of cytokinin (BA: 5-10 mg L^{-1}) were prerequisite for inducing adventitious shoots, which was very different from our result. We observed that high concentration of BA (over 3 mg L^{-1}) resulted in very low shoot differentiation and low concentration of IAA (0.2 mg L^{-1}) was helpful for shoot induction and differentiation. Besides, a higher concentration of auxin inhibits shoot formation and promotes callus proliferation (Tabei et al., 1991).

In this study, the shoot formation frequency of the three cultivars was different between the basal region and apical

region of cotyledon. The basal region had higher frequency (79.17-83.33%) than the apical region (5.23-8.25%) after four weeks culture on regeneration medium. Similar results were reported by Monacelli et al. (1988), Tabei et al. (1993) and Košmrlj et al. (2015). These phenomena suggested that the proximal end of each cotyledon segment regenerated adventitious shoots more effectively than the distal end and the basal region showed polarity. Compton and Gray (1993b) obtained adventitious shoots only on the proximal region of cotyledons and suggested that the competence for adventitious shoot formation in watermelon was restricted to the proximal region of the cotyledons. Besides, it had the highest regeneration rate (89.67%) when entire cotyledon was used as explants (Li et al., 2011). However, Choi et al. (1994) indicated that the distal half cotyledonary explants produced more shoots than the proximal half. They thought cells competent for shoot formation are not localized at one site of the cotyledon in these cultivars and light was essential for adventitious shoot formation, because no cotyledonary explants of two cultivars ('Sweet Gem' and 'Gold Medal') formed shoots in the dark (Choi et al., 1994). In this study, many shoots appeared at the proximal end of the basal region. Our results supported that the proximal end of basal region had high potential for shoot formation.

The age of donor seedlings is one of the key factors for adventitious bud induction in *C. lanatus* sp. and *Cucurbita* sp. (Zhang et al., 2008). The numerical results indicated that cotyledon from 5-day-old seedlings were the most sensitive to shoot formation (Dong and Jia, 1991; Choi et al., 1994; Pirinç et al., 2003) and similar results were obtained in our study. However, different optimal age of cotyledons for adventitious bud induction had been reported; such as two days (Compton and Gray, 1993c), three days (Tabei et al., 1993; Ellul et al., 2003; Krug et al., 2005; Yu et al., 2011), seven days (Huang et al., 2011) and 7-10 days (Cho et al., 2008). A possible explanation is that type and concentration of disinfectant, sterilization time and seed storage time could affect the physiological status of mature seeds.

Although natural aromatic cytokinins such as BA and kinetin were most commonly used in regeneration systems, it might have several side effects, such as difficulties in rooting, hyperhydricity, stunted shoots and callus formation (Magyar-Tábori et al., 2010). The undesirable effects are attributed to either its N7- and N9-glucosylation or to conjugation with alanine, which results in biologically inactive but chemically stable derivatives and enabling slow release of active compounds (Werbrouck et al., 1995). In this study, we also found that there was hyperhydricity phenomenon in watermelon tissue culture, but lower concentration of BA can reduce the number of hyperhydric adventitious shoots. Yu et al. (2011) indicated that SH vitamins with 50 mg L^{-1} thiamine HCl could overcome the hyperhydric phenotype and effectively reduce the percentage of hyperhydricity in watermelon. However, the reason SH vitamins affected hyperhydricity is still not clear. Besides, it has been reported that hyperhydricity would be prevented by adding silicon to the culture medium (Sivanesan et al., 2010) and Ag^{+} (Vinoth and Ravindhran, 2015), decreasing the ratio of NH_4^+: NO_3^- (Ivanova and Van Staden, 2009), increasing agar concentration (Brand, 1993), and using techniques such as bottom cooling (Saher et al., 2005).

Watermelon regenerated seedling is prone to etiolation and senescence, which might be caused by rapid growth of many adventitious buds. Through tests we found that shortening subculture times could get good regeneration plants. Another noteworthy result is that the multiple shoots regenerated from watermelon explants must be transferred to elongation medium, because prolonged culture on shoot induction medium not only stimulates callus formation but also produces abnormal shoots. Similar phenomenon was found in other watermelon cultivars (Chaturvedi and Bhatnagar, 2001). One of the most commonly used selection marker genes for screening transgenic plants is *npt*II, which encodes a phosphotransferase capable of phosphorylating aminoglycoside antibiotics, including kanamycin, geneticin, neomycin and paromomycin (Yoshikura, 1989). To date, kanamycin has been commonly used for the selection of *npt*II transformed plants of watermelon (Huang et al., 2011; Yu et al., 2011) and melon (Choi et al., 2012). The optimal concentrations of kanamycin used to suppress non-transgenic watermelon adventitious shoots were different, such as 40 mg L^{-1} (Park et al., 2005), 100 mg L^{-1} (Choi et al., 1994; Huang et al., 2011; Yu et al., 2011), 125-175 mg L^{-1} (Ellul et al., 2003) and 200 mg L^{-1} (Huang et al., 2011; Yu et al., 2011). Similar phenomenon was reported by Choi et al. (2012). Our results indicated that differentiation of adventitious buds was suppressed with 100 mg L^{-1} kanamycin. Thus, 100 mg L^{-1} kanamycin proved to be the optimal concentration for screening the transformants.

In conclusion, our results clearly demonstrate that high frequency *in vitro* plant regeneration of watermelon can be obtained by the proper combination of explant age, types and concentrations of plant growth hormones. The system will be potentially useful in the transformation of watermelon via *Agrobacterium*-mediated or micro particle bombardment.

Conflict of interests

The authors did not declare any conflict of interest.

ACKNOWLEDGEMENTS

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